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THE COUNCIL FOR TOBACCO RESEARCH–U.S.A. INC.

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JUN 27 1975

Application for Research Grant
(Use extra pages as needed)

Date: June 23, 1975

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3. Department(s) where research will be done or collaboration provided:

Department of Pathology

4. Short title of study:

Biology of Suppressor T Cells

5. Proposed starting date: January 1, 1976

6. Estimated time to complete: 3 years

7. Brief description of specific research aims:

The understanding of physiologic mechanisms which regulate immune responses is a prerequisite for understanding the pathogenesis of disease processes with immunologic components and the effects of environmental factors, such as cigarette smoking, on the immune mechanism. We propose to investigate the mechanisms by which suppressor T cells and their products regulate the development and expression of humoral and cell-mediated immune responses in tissue culture systems. Initially, concanavalin A-activated suppressor T cells and the biologically active mediators they secrete will be used to probe non-antigen-specific regulatory mechanisms. Experimental systems for activation of antigen-specific suppressor T cells and secretion of antigen-specific factors will be developed. These cells and their products will be used in various experimental situations to define the critical parameters which permit selective suppression of a) humoral immune responses without compromising cell-mediated immune responses to the same antigenic determinants, and b) the reverse situation, suppression of cell-mediated immune responses without affecting antibody responses. After these parameters have been defined in tissue culture systems, this information will be applied to relevant animal models.

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8. Brief statement of working hypothesis:

Activation of the complex, multicellular immune mechanism of mammals by foreign antigen results in the development of humoral and cell-mediated immune responses which may either protect the host from invasion by infectious organisms and neoplastic cells or be responsible for rejection of potentially beneficial tissue transplants and the pathogenesis of several disease processes (1). Cigarette smoking has been implicated in the etiology and pathogenesis of a number of respiratory diseases in man (2). Further, cigarette smoking in man and chronic exposure of laboratory animals to cigarette smoke has been associated with impaired humoral (3-9) and cell-mediated (10,11) immune responses and decreased phagocytic and bacteriocidal activity of alveolar macrophages (12-16). The cells of the immune system survive poorly after acute exposure to cigarette smoke *in vitro* (17), but chronic exposure *in vivo* leads to an accumulation of alveolar macrophages resistant to the toxic effects of tobacco smoke but with undetermined functional capacity (8,16,18). However, these effects of cigarette smoke on the cells and responses of the immune system cannot be adequately evaluated until the immune mechanism itself is better understood. This proposal is directed to gaining a better understanding of one aspect of the immune system: the physiologic mechanisms which regulate development and expression of immune responses.

9. Details of experimental design and procedures (append extra pages as necessary)

RESEARCH PROPOSAL

I. Background Information

A. Studies of Other Investigators. Two distinct types of antigen-specific lymphocytes are the precursors of the immune effector cells. In humoral immune responses, the precursors of antibody-producing cells, B cells, respond to antigenic stimulation by differentiating into plasma cells which secrete antibody molecules specific for the stimulating antigen (1, 19, 20). In cell-mediated immune responses, thymus-derived lymphocytes, T cells, after stimulation by cell membrane antigens, may develop into cytotoxic lymphocytes which mediate the various rejection phenomena (allograft and tumor rejection, and graft-versus-host responses) (1, 21-23). Antigen or mitogen activated T cells may also secrete a variety of biologically active mediators (including macrophage migration inhibitory factor (MIF), chemotactic factors, lymphotoxins, skin reactive factors, and interferon) which play a part in the inflammatory processes of delayed hypersensitivity reactions, cytotoxicity to tumor cells, resistance to certain infectious organisms, and activation of macrophages (1, 24-26). In addition, both T and B cells may be rendered tolerant or unresponsive after interaction with antigen under appropriate circumstances (27).

A third, non-specific accessory cell, the macrophage, has important functions in uptake, catabolism and presentation of antigen to T and B cells so crucial in the initiation of immune responses and avoidance of tolerance induction (28). These cells may also act as non-specific effector cells in the expression of both humoral and cell-mediated immune responses (24-26, 28).

T cells are also the critical regulators of the development and expression of both cell-mediated and humoral immune responses (1, 20, 23, 29-31). In a positive regulatory capacity, T cells may function as "helper cells" for development of antibody responses by B cells to complex, multideterminant antigens (T cell-dependent antigens) (1, 20, 29-31), or as "amplifier cells" for development of cell-mediated immune responses, such as graft-versus-host and cytotoxic lymphocyte responses, by other T cells (22, 23, 30, 31). In a negative regulatory capacity, suppressor T cells have been implicated in the regulation of development and expression of most T cell and B cell responses to antigen (23, 27, 29-31). The ubiquitous nature of suppressor T cells is illustrated

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by their involvement in: a) many of the phenomena of immunologic tolerance (27); b) antigenic competition (32); c) regulation of IgE antibody responses (33); d) regulation of antibody responses to "T cell-independent antigens" (34); e) chronic allotype suppression (35); f) regulation of antibody responses controlled by histocompatibility-linked immune response genes (36); g) regulation of mixed lymphocyte, cytotoxic lymphocyte, and graft-versus-host responses (23); h) regulation of development and expression of delayed hypersensitivity responses (27, 29-31, 37-39); and i) regulation of development of autoimmune diseases (40, 41).

Suppressor and helper T cells appear to be functionally distinct subsets of T cells rather than a single population of cells whose regulatory function reflects a critical stage of activation (23, 27, 29-31, 42). Suppressor T cells appear to be relatively immature cells which have abundant θ antigen and are functionally short-lived, spleen-seeking, and, in many instances, radiosensitive. In contrast, helper T cells appear to be more mature cells which have less θ antigen and are functionally long-lived, lymph node-seeking, and radioresistant, at least after priming with antigen.

The regulatory activities of T cells may be antigen-specific, e.g., carrier-specific helper or suppressor functions (20, 27, 29-31, 33, 43), or, although T cells are activated by specific antigen, their effects on immune responses may be non-specific, e.g., antigenic competition and the "allogeneic effect" (27, 29-32, 44-46). Lastly, phytomitogens non-specifically activate T cells whose enhancing or suppressing effects on immune responses are also non-specific (42, 47-50). Soluble products released by activated T cells mediate the regulatory functions in some immune responses (20, 27, 29-33, 43-46, 50-52), whereas regulation of other responses may require direct cell-to-cell contact (20, 23, 27, 29-31, 49). Some of the soluble products of activated T cells have been partially characterized. In one system, the mediator of both helper and suppressor functions appears to be a monomeric IgM-like molecule, IgT, produced by T cells (52); a similar molecule appears to mediate only carrier-specific helper functions in another system (33). Further, other investigators have found that T cell factors with different physicochemical properties are involved in the non-specific enhancement of IgG and IgE antibody responses (46). Another class of mediators appears to be a product(s) of the K and/or I regions of the major histocompatibility complex (33, 43, 45, 53). These mediators may lack antigen specificity (45, 53), or have specificity for the carrier moiety of the immunogen (33, 43) and mediate both helper (43, 45, 53) and suppressor functions (33) in different experimental systems. T cells or their products may act directly on potentially responsive T or B cells, or indirectly on macrophages by mechanisms which are still altogether unclear (23, 27, 29-31, 33, 46, 52).

Although much of the phenomenology of helper and suppressor T cells has been recognized and described, our understanding of the precise mechanisms by which these cells and their products operate is still quite obscure.

B. Studies from our Laboratory. Several years ago, experiments were initiated to investigate the immunological consequences of T lymphocyte activation. Since antigen activates only those relatively few T cells having membrane receptors specific for that antigen, the plant lectin and mitogen concanavalin A (Con A) was used to non-specifically activate larger numbers of T cells. Primary IgM and IgG plaque-forming (PFC) responses to sheep erythrocytes (SRBC) by mouse spleen

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cells *in vitro* were profoundly suppressed by mitogenic concentrations (1 µg/ml) of Con A added at culture initiation (54). In addition, mitogenic concentrations of Con A suppressed the generation of cytotoxic lymphocytes (CL) in mixed lymphocyte cultures (MLC) of mouse spleen cells (49). The precise mechanisms by which Con A affects immune responses remain to be determined, but multiple mechanisms appear to be involved (55). However, the observation that spleen cells from mice injected with Con A suppressed PFC repsonses by normal spleen cells *in vitro* (54) prompted us to investigate whether one mechanism of Con A-mediated suppression was the activation of a population of suppressor T cells.

Small numbers of mouse spleen or lymph node cells, but not thymus cells, after incubation with 1 µg of Con A/ml for 48 hrs, profoundly suppressed PFC responses to SRBC(48), GAT(synthetic random terpolymer of L-glutamic acid^{60}-L-alanine^{30}-L-tyrosine^{10}), and the T cell-independent antigens, DNP-Ficoll and TNP-lipopolysaccharide (56), and CL responses to alloantigens generated in MLC (49) by normal spleen cells. Other investigators have shown that Con A-activated spleen cells suppress DNA synthetic responses in MLC (57) and, depending on the experimental circumstances suppress or enhance PFC responses *in vitro* (42, 47, 50, 58, 59). The suppression of these immune responses was not antigen-specific and was not mediated by transferred Con A or non-specific cytotoxicity on responding spleen cells as determined by viable cell recovery. The suppression by Con A-activated spleen cells was mediated by T cells, since treatment of spleen cells with anti-θ serum and complement, either before or after activation with Con A, eliminated suppressor cell activity (48, 49). Further, X-irradiation (2000 R) of spleen cells before activation with Con A abrogated generation of suppressor T cells, whereas after activation, the function of suppressor T cells was radioresistant. Suppression of PFC and CL responses was critically dependent on both the numbers of Con A-activated cells added and the time of addition to the responding cultures. Decreasing the number of Con A-activated cells below 10% of the number of responding spleen cells resulted in progressively less suppression, whereas increasing the number over 20% often resulted in non-specific cytotoxicity. Suppression of PFC responses was observed when suppressor T cells were added during the first 48 hrs of culture, whereas CL responses were most efficiently suppressed when Con A-activated cells were added during the first 24 hrs of culture (48, 49).

The most interesting aspect of the suppression of PFC and CL responses by Con A-activated suppressor T cells, and one which may provide a clue to their mechanism(s) of action, is their effect on the kinetics of development of these responses. Kinetic analysis of PFC responses to SRBC revealed that the 90% or greater suppression observed on days 5 and 6 in cultures to which Con A-activated suppressor T cells were added at initiation was not due to a failure to initiate the PFC response. On days 2 and 3 of culture, PFC responses in cultures containing suppressor cells were the same or slightly greater than responses in cultures to which non-Con A-activated control spleen cells were added. The PFC responses in cultures containing the suppressor cells aborted dramatically, however, after 72 hrs, at the time when the PFC responses in control cultures was expanding exponentially. From days 4 to 6 when PFC responses in control cultures were maximum, PFC responses in cultures containing suppressor cells fell precipitously (51). A similar pattern of suppression was observed in CL responses generated in MLC; on days 3 and 4, CL responses in control cultures and cultures to which suppressor

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cells in vitro were profoundly suppressed by mitogenic concentrations (1 µg/ml) cells had been added at initiation were similar. On days 4 through 6, CL responses in control cultures increased progressively, however, during this interval, a definite and continued inhibition of CL responses was observed in cultures with suppressor cells (49). The dichotomy between the time early in the response when suppressor T cells must be present to achieve suppression and the later time during expression of maximum responses in control cultures when suppression of PFC and CL responses was actually manifested led to experiments investigating the mechanisms involved. a population of suppressor T cells.

In these experiments, we found that supernatant fluids from cultures of Con A-activated spleen cells, from which residual Con A had been removed by Sephadex absorption, contained factors, termed soluble immune response suppressors (SIRS), which suppressed PFC responses without cytotoxicity on responding spleen cells. Kinetic analysis of PFC responses revealed a pattern of suppression identical to that observed with suppressor cells (51). However, to date we have been unable to suppress CL responses with SIRS (30, 31, 60); the reasons for this failure require further experimentation which may provide additional information about the mechanisms of action of Con A-activated suppressor T cells and SIRS.

SIRS activity was detected in supernatant fluids of Con A-activated spleen cells within 6 hrs, and was maximal in fluids harvested between 12 and 48 hrs after initiation (51). Interestingly, the kinetics of secretion of SIRS is similar to that of mitogen-induced secretion of the T cell mediators MIF (61) and interferon (62). Supernatant fluids with SIRS activity also have MIF activity; in all of the analyses to date we have been unable to dissociate definitively SIRS and MIF activity (31, 63). Physicochemical characterization has shown that SIRS is a heterogeneous glycoprotein with a molecular weight in the range of 35,000 to 68,000 daltons which is soluble in 70% $(NH_4)_2SO_4$ and stable at 56°C for 60 min, but destroyed in 10 min at 70°C and at pH 2. SIRS is not absorbed by antigen, mouse immunoglobulins, or antisera against mouse immunoglobulins or histocompatibility antigens, but is absorbed by spleen cells from several mouse strains. SIRS also has no strain specificity in its effects (31, 63). Further, SIRS mediates its suppression by acting on macrophages and not T cells or B cells, and, like MIF, can be blocked by L-fucose, adding further support to the notion that SIRS and MIF may be the same molecules (31, 64).

Suppressor T cells and their products generated by activation of T cells with Con A provide a viable experimental system to probe the biology of suppressor T cells. Many experiments are needed to characterize these suppressor T cells and SIRS more precisely and to determine their mechanism(s) of action in suppression of PFC responses, and why SIRS fails to suppress CL responses. These experiments will be described in Section 3.

However, Con A-induced suppressor T cells and SIRS have the disadvantage of being non-antigen specific in their effects. Preliminary observations from our laboratory indicate that antigen-specific suppressor T cells can be generated in tissue culture in certain circumstances. First, T cells harvested from MLC on days 6 or 7, after the peak of a CL response, specifically suppress generation of CL responses to the same alloantigens by normal syngeneic cells. Second, T cells harvested from a culture after the peak of a PFC response specifically suppress

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PFC responses to the same antigen by normal spleen cells. The detailed investigation of these preliminary observations is the basis for part of this application.

II. Specific Aims. The specific aims of this project have been divided into two groups:

A. Further characterization of Con A-activated suppressor T cells and their products (SIRS)

1. Investigation of the metabolic activities required for activation of suppressor T cells, secretion of SIRS, and expression of biological activity by these T cells.
2. Further physicochemical characterization of SIRS and comparison with other T cell mediators.
3. Investigation of the effects of SIRS on the functions of macrophages and evaluation of secondary effects of SIRS-activated macrophages on the functions of T cells and B cells.
4. Investigation of the reasons for the failure of SIRS to suppress CL responses.

B. Development of experimental systems for generation of antigen-specific suppressor T cells and their products

1. Definition of parameters for generation of antigen-specific suppressor T cells and their soluble products.
2. Physicochemical characterization of active T cell products and comparison with Con A-induced mediators.
3. Determination of the cellular site(s) of action and mechanism(s) by which these T cells and their products suppress immune responses.
4. Development of an experimental model which allows antigen-specific suppression of antibody responses without affecting cell-mediated responses to the same antigen and vice versa.

III. Methods of Procedure. The experimental systems necessary for investigating the mechanisms by which suppressor T cells and their products regulate immune responses are routine procedures in this laboratory. Initially tissue culture systems will be used exclusively, but in later stages of the project *in vivo* animal models will be used.

Culture and Assay Systems. Antibody responses will be generated in cultures of mouse spleen cells using the system of Mishell and Dutton (65) as modified in our laboratory (66). Stimulating antigens will be sheep erythrocytes (SRBC), the synthetic polypeptide antigen GAT, and mouse alloantigens coupled to pigeon erythrocytes. IgM and IgG PFC responses will be measured by the hemolytic plaque technique using SRBC or the determinant of interest coupled to SRBC (66, 67). Cytotoxic lymphocytes (CL) responses will be generated by incubating responder spleen cells with appropriate mitomycin C-treated allogeneic spleen cells in Mishell-Dutton type cultures. CL responses will be measured in the standard ^{51}Cr release assay using P815 mastocytoma or EL-4 leukemia as target cells (49). A recently developed system which allows the generation of simultaneous and non-cross-reactive CL responses to two sets of alloantigens in MLC (68) will be used in the studies of antigen-specific suppressor T cells and their products.

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Generation of Suppressor T Cells and SIRS by Con A. Suppressor T cells will be generated by incubating spleen cells with Con A (1 µg/ml) for 48 hrs. After

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separations of the cells, supernatant fluids will be absorbed with Sephadex G75 to remove residual Con A; the cells will be washed three times with medium. Control spleen cells will be incubated for 48 hrs without Con A, which will be added at the time of harvest of the cultures; thereafter, these cells and supernatant fluids will be washed or absorbed as described for the Con A-activated preparations (31, 48, 49, 51, 63). Since SIRS is soluble in 70% $(NH_4)_2SO_4$, crude supernatant fluids will be purified initially by precipitation of irrelevant material by 70% $(NH_4)_2SO_4$. Further treatment of the cells, purification procedures for SIRS, and the preparation, testing, and purification of antigen-specific suppressor T cells and their products will be described in later sections. [illegible] SIRS, and expression of biological activity by these T cells.

Specific Experimental Protocols. [illegible]

A. Further characterization of Con A-activated suppressor T cells and their products (SIRS).

[illegible] on the function of T cells and B cells.

1. Investigation of the metabolic activities required for the activation of suppressor T cells, secretion of SIRS, and expression of biological activity by these T cells. Previous studies have shown that X-irradiation (2000 R) of spleen cells prior to activation with Con A abrogates generation of suppressor T cells, but after activation, suppressor T cell function is radioresistant (48, 49). Further experiments will determine whether X-irradiated T cells stimulated with Con A can secrete SIRS (tested on PFC responses). In addition, the effects of inhibitors of DNA synthesis (hydroxyurea or mitomycin C), RNA synthesis (chromomycin A_3 or actinomycin D) and protein synthesis (cycloheximide or puromycin) on generation of Con A-activated suppressor T cells (tested on PFC and CL responses) and secretion of SIRS (tested on PFC responses) will be determined. It is anticipated that inhibitors of protein synthesis and probably RNA synthesis will block production of SIRS and that inhibitors of DNA synthesis will block generation of suppressor T cells. These same inhibitors, plus colchicine and cytochalasin B (to disrupt microtubules) and antimycin A (to inhibit oxidative phosphorylation) will be used to determine which metabolic activities are required for expression of the biological activity of suppressor T cells (tested on PFC and CL responses). Inhibitors of DNA synthesis are expected to be without effect in this regard. Other metabolic inhibitors will be used if indicated by these studies. This analysis will be useful in determining whether suppressor T cells mediate their effects on immune responses only by secretion of SIRS or whether some other activities mediated directly by the cells are responsible. This determination is crucial in analyzing why SIRS does not suppress CL responses (See Section A.4 below).

[illegible] synthetic polypeptide antigen GAT, and mouse alloantigens could be present.

2. Physicochemical characterization of SIRS and comparison with other T cell mediators. Supernatant fluids from cultures of Con A-activated spleen cells have SIRS and MIF activity. In all of the separatory and analytical procedures used, including column chromotography on Sephadex G100, polyacrylamide gel electrophoresis, CsCl density gradient ultracentrifugation, treatment with proteolytic enzymes and absorption with a variety of insoluble immunologically relevant materials, we have been unable to dissociate MIF and SIRS activity (31, 63). In addition, these supernatant fluids should have interferon activity (62), another T cell mediator which inhibits PFC responses *in vitro* in a manner analogous to SIRS (69). However, SIRS is labile at pH 2, suggesting it is not interferon Type I (69). Younger and Salvin (70) have described a Type II interferon which is

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also labile at pH 2 but otherwise indistinguishable from mouse MIF. Thus, SIRS will be analyzed for interferon activity by standard methods (71); we expect to find interferon activity in these preparations. After each future purification or treatment procedure, the preparations will be analyzed for SIRS, MIF, and interferon activity in an attempt to distinguish among these mediators. Dr. Salvin has agreed to exchange reagents with us in an attempt to resolve this issue.

Further purification and characterization procedures will be aided by internally labeling SIRS with ^{14}C amino acids and by precipitation of irrelevant material with 70% $(NH_4)_2SO_4$. Since SIRS is a glycoprotein, based on CsCl density gradient ultracentrifugation (31, 63), it will be purified further by passage over a Con A-Sepharose column; non-bound and methylmannoside elutable material will be assayed for biological activity. Further characterization will involve determining the sensitivity of the various biological activities to the action of several glycosidases, including neuraminidase, L-fucosidase, B-galactosidase, and N-acetylglucosaminidase (72). This analysis will identify which, if any, of the sugar moieties in the glycoprotein are necessary for its biological activities.

L-fucose partially inhibits the ability of SIRS to suppress PFC responses (30, 64). The capacity of various other sugars (72), including sialic acid and several hexoses, hexosamines, and N-acetylhexoamines, to competitively inhibit or absorb SIRS, MIF, and interferon activity will be investigated. In absorption studies, SIRS will be passed over Sepharose columns to which the sugar moieties have been coupled in an insoluble form and the effluent will be assayed for biological activity. For competitive inhibition studies with the sugars, advantage will be taken of the fact that SIRS acts on macrophages and that exposure of macrophages to SIRS for 2 hrs is sufficient to achieve suppression of the PFC responses generated by T and B lymphocytes added to the treated macrophages (31, 64). Thus, separated macrophages can be treated with SIRS in the presence of the various sugars and washed before addition of lymphocytes, thus avoiding the non-specific toxic effects of some of the sugars on lymphocytes. Modifications of this approach will be used in assays for MIF and interferon activity. This analysis will determine which sugars, in addition to L-fucose, are involved in the binding of SIRS to macrophages and other biological activities of SIRS, e.g. MIF and interferon activity.

3. Effects of SIRS on functions of macrophages and evaluation of secondary effects of SIRS-activated macrophages on T cells and B cells. SIRS acts on macrophages, but does not block initiation of the PFC response (31, 64). Instead, after developing normally during the first 3 days of culture, PFC responses abruptly abort and the number of PFC decrease precipitiously on days 4 through 6 (30, 31, 51). Furthermore, macrophages treated with SIRS have no obvious defects in uptake, catabolism, or retention of the soluble antigen, GAT (64). SIRS may interfere with functions of macrophages essential later in the PFC response, or SIRS may activate macrophages so that they or their products are actively inhibitory. Since macrophages are not required in the cultures after 48 hrs incubation for development of normal PFC responses (73), the latter alternative seems more likely at this time. Initially, the effects of SIRS on the morphology and accessory functions of macrophages will be evaluated. Morphological changes, such as increased spreading of cytoplasmic processes on the culture dish, "ruffling" of the plasma membrane and increased numbers of cytoplasmic lysosomes, all of which are characteristic of "activated macrophages" (25, 26, 72) are expected and will be surveyed

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also labile at pH 2 but otherwise indistinguishable from mouse MIF. The SIRS with phase or scanning electron microscopy. The loss of membrane proteoglycan residues, which is characteristic of MIF-activated macrophages, will be evaluated by staining SIRS-treated and untreated macrophages with ferrocyanide reduced osmium tetroxide (74). Functions characteristic of activated macrophages, such as increased phagocytic capability, cytotoxicity or cytostasis of tumor cells, and increased bactericidal activity (25, 26, 72, 75) will be evaluated. This analysis will provide information on the effects of SIRS on other physiologic functions of macrophages. [illegible] amino acids and by precipitation of irrelevant material with [illegible]. Since SIRS is a glycoprotein based on [illegible] grade. The possibility that SIRS-treated macrophages elaborate low molecular weight products, similar to those shown in other systems to inhibit lymphocyte or tumor cell proliferation (75) will be determined, as well as their ability to secrete collagenase (77). If these macrophages elaborate inhibitory low molecular weight products, the products, their target cells, and mechanisms of action will be determined. If these macrophages elaborate collagenase, the effects of this enzyme on PFC responses and its relevance to SIRS-induced inhibition of PFC responses will be determined. In conjunction with the studies on production of factors by macrophages, the rates of DNA, RNA, and protein synthesis at specific intervals in suppressed cultures, as determined by incorporation of ^{3}H-thymidine, ^{3}H-uridine, and ^{3}H-leucine, respectively, will be correlated with the abortion of the PFC response after day 3 of culture. This analysis will reveal whether the abortion of the PFC response is due to cessation of proliferation of precursors of PFC, or cessation of antibody synthesis by these cells which continue to proliferate at a normal rate.

Treatment of macrophages with proteolytic enzymes (trypsin or chymotrypsin) and various glycosidases (fucosidase, neuraminidase, and N-acetylglucosaminidase) (72) before reaction with SIRS should provide information as to what moieties SIRS interacts with on macrophage membranes. The effects of these enzymes on the function of macrophages in PFC responses will be determined for control purposes. These results will be correlated with the results of experiments described in Section A.2 and should provide information about the receptor sites on both macrophages and SIRS which are critical for SIRS-induced suppression of PFC responses. Further, membrane esterases inhibit the effect of MIF on macrophages (72). Working on the assumption that MIF and SIRS activity are not dissociable, the effects of inhibiting this esterase activity by diisoflurophosphate or α_1 anti-trypsin (25, 26, 72) on the SIRS-induced inhibition of PFC responses will be determined. This analysis will be particularly useful when applied to peritoneal exudate macrophages, which are much less susceptible to the effects of SIRS than splenic macrophages (64). It is conceivable that peritoneal macrophages have higher levels of membrane esterase activity than splenic macrophages and that this accounts for the inability of SIRS to mediate its effect on peritoneal macrophages. It is anticipated that peritoneal macrophages will be susceptible to effects of SIRS after inhibition of membrane esterases.

4. Analysis of why SIRS fails to suppress CL responses. These experiments may be unnecessary if the experiments described in Section A.1 show that suppressor T cells regulate immune responses by mechanisms other than the secretion of SIRS.

If this series of experiments is necessary, the effects of SIRS on T cell proliferative responses to Con A, PHA, and allogeneic cells measured by ^{3}H-thymidine

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incorporation will be determined to evaluate the ability of SIRS to suppress other T cell responses.

In the MLC, biologically active products are elaborated which enhance immune responses (45, 78); these products may override the suppressive effects of SIRS in the generation of CL responses. To test this possibility, soluble products from an MLC will be added in varying concentrations with SIRS to cultures of spleen cells stimulated with SRBC to determine whether the SIRS-mediated suppression of the PFC response is overriden by MLC products. This indirect test should provide evidence as to whether a similar mechanism accounts for failure of SIRS to inhibit CL responses.

Lastly, the larger numbers of macrophages in cultures used to generate CL responses may circumvent SIRS-mediated suppression. Addition of an excess of macrophages to SIRS-treated cultures often overcomes suppression of PFC responses (64). Thus, the number of macrophages in cultures used to generate CL responses will be reduced as much as possible without compromising the CL responses and the effects of SIRS in these cultures will be evaluated.

However, from our experiences with these experimental systems, it is our expectation that SIRS will be unable to suppress CL responses, and that these responses will be suppressed by Con A-activated suppressor T cells by mechanisms other than the activity of SIRS. These mechanisms will be probed first by determining the target cell(s) (macrophages, T cells, or B cells) of the suppressor T cells and then by determining the specific effects of these cells on the target cell(s). If this analysis is necessary, we anticipate that it will be one of the most difficult portions of the project.

B. Development of experimental systems for generation of antigen-specific suppressor T cells and their products.

1. Definition of parameters for generation of antigen-specific suppressor T cells and their soluble products. This portion of the project is crucial to the objective of developing experimental systems which allow selective suppression of antibody responses without affecting cell-mediated immune responses to the same antigen and vice versa. Our observations with the generation of antigen-specific suppressor T cells and their products are preliminary; because of our relative ignorance about these systems, this portion of the application will, of necessity, be less specific and more tentative. However, we intend to take full advantage of lessons learned in studies with Con A-induced suppressor T cells and SIRS and apply them in this portion of the project. The observations that antigen-specific suppressor T cells can be recovered from cultures after the peak of either a PFC response to SRBC or a CL response to allogeneic cells provide the starting point for this project.

The culture conditions for recovery of optimal suppressor T cell activity after the peak of a PFC or CL response will be determined initially. The T cells will be purified on appropriate immunoadsorbent columns (36, 79), identified as T cells by sensitivity to anti-θ serum and complement, and tested for their ability to specifically suppress PFC or CL responses to the antigens which stimulated their generation. These T cells will also be incubated with specific or irrelevant antigen to induce production of biologically active soluble products. If such products cannot be recovered in culture supernatant fluids, the cells will be

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sonicated to release these products (80), which, after ultracentrifugation to remove cell debris, will be tested for specific suppressive activity on PFC and CL responses. The rationale for this approach is derived from the observations that suppressor T cells or their products normally act physiologically to limit the duration and/or magnitude of immune responses, i.e. this is a normal physiological homeostatic mechanism (29-31).

Another approach will be to incubate suspensions of T cells (thymocytes, cortisone resistant thymocytes, or purified peripheral T cells) with appropriate antigen for various periods of time, either with or without macrophages. The cells and supernatant fluids (after removal of any residual antigen by immunoabsorbent columns) will be tested for specific suppressive activity on PFC and CL responses.

These experiments will provide useful information about the generation and biology of antigen-specific suppressor T cells and their biologically active products in general. However, one major thrust of this project is to generate suppressor T cells and/or their products which will specifically suppress antibody responses to mouse alloantigens without compromising cell-mediated immune responses to cells bearing the same alloantigens. Thus, alloantigens in membranes of lymphocytes from appropriate mouse strains will be internally labeled with ^{35}S methionine or trace labeled with ^{125}I using the lactoperoxidase method (81) and extracted with NP40 or other suitable detergents (82). The desired alloantigens, products of the H-2 gene complex, will be purified on appropriate immunoadsorbent columns of insolubilized anti-H-2 sera (83). CL responses to these alloantigens will be generated by incubating responder spleen cells with appropriate mitomycin C-treated allogeneic spleen cells in the usual fashion. Soluble alloantigens will be coupled to pigeon erythrocytes with carbodiimide (84) for stimulation of PFC responses in Mishell-Dutton cultures. PFC responses will be measured in the hemolytic plaque assay using the alloantigens coupled to SRBC as indicator cells. The development of this system will permit determination of the critical parameters for specific suppression of PFC responses without affecting CL responses to the same antigens using the antigen-specific suppressor T cells and their products generated as described above. The system for generating simultaneous non-cross-reactive CL responses to two sets of alloantigens (68) will be especially useful in this portion of the project.

2. Physicochemical characterization of active T cell products.

Antigen-specific suppressor T cell products will be purified and characterized by the same battery of techniques which have been used with SIRS. Sephadex G100 and G200 column chromatography, polyacrylamide gel electrophoresis including SDS and isoelectric focusing gels (85), and CsCl density gradient ultracentrifugation will be used to determine molecular weight, electrophoretic mobility, relative carbohydrate content, and molecular chain composition. Sensitivity to temperature, proteolytic enzymes, glycosidases, and nucleases will also be determined to further characterize the active molecules. The nature of the receptors on these molecules for cells or antigen will be evaluated by determining the ability of macrophages, T cells and B cells and specifically reactive columns (83) to absorb the biological activity. Alloantigen, mouse immunoglobulin, various sugars, Con A, anti-immunoglobulin, and anti-histocompatibility antigen columns will be used initially. Other specifically reactive columns will be used as dictated by the results of these studies. These T cell products will also be evaluated for MIF and interferon activity. Further purification and characterization will be employed as needed. After each procedure, the preparation will be tested for biological activity on PFC and CL responses to mouse alloantigens. The characterization of these molecules is

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a critical first step in understanding the mechanisms of their biological activity. It is anticipated that these products may be identified as products of the major histocompatibility or H-2 gene complex (33, 43, 45, 53).

3. Determination of cellular site(s) and mechanism(s) of action of antigen-specific suppressor T cells and their products. This portion of the project will be greatly simplified if we have succeeded in obtaining an antigen-specific T cell factor which suppresses antibody responses, but not CL responses, and the reverse. In this case, the minimum exposure time of responding spleen cells to the factor required to suppress PFC responses, for example, to a given antigen, will be determined. Then purified macrophages, T cells and B cells will be exposed to the factor and cultures containing all the possible combinations of treated and normal cells will be evaluated to determine the target cells of the factor. A similar approach will be used to determine the target cell for suppression of CL responses. Depending on the results of these analyses, the effects on functions which the target cell usually performs in the immune response will be analyzed. For example, the effects on a) antigen presentation functions of macrophages, b) cooperative interactions of T cells with B cells, and, c) actual antibody production by B cells will be determined. The determination of the effects of suppressor T cells or their products on the kinetics of development of PFC and CL responses will be especially important. Failure to initiate a response suggests an effect on macrophages, whereas suppression of the responses with a pattern similar to that observed with SIRS would indicate that some event after successful initiation of the response has been affected. PFC and CL responses and DNA, RNA, and protein synthesis in suppressed cultures will also be correlated to determine the mechanism of action of the factor. Further analyses will be carried out depending on the results of these experiments.

If suppressor T cells are required to achieve the desired effects, a similar pattern of attack will be employed, but the analysis will be considerably more complicated, as indicated above for the site(s) and mechanism(s) of action of Con A-activated suppressor T cells.

4. Development of the experimental model. If this project has developed successfully using the approaches outlined above, we will be able to specifically suppress antibody responses to an antigen without affecting cell-mediated immune responses to the same antigens and vice versa. This experimental system will be developed and defined using tissue culture systems. Once operational, the experimental system will be used to study regulation of antibody and cytotoxic lymphocytes responses *in vivo*. Eventually, this system could be used to probe regulatory mechanisms in response to tumor-specific antigens.

IV. Significance

The significance of various experimental procedures and anticipated results has been pointed out in the descriptions of methods of procedure.

The understanding of mechanisms which regulate development and expression of humoral and cell-mediated immune responses is crucial for evaluating the effects of environmental factors, such as cigarette smoking, on the cells and responses of the immune mechanism. Further, the understanding of these regulatory mechanisms should permit manipulation of the immune mechanism for alleviation of disease processes with immunological components.

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This project is designed in part to investigate in detail the mechanisms by which mitogen-induced, non-antigen-specific suppressor T cells and their secreted mediators (SIRS, MIF, ?interferon) regulate development and expression of humoral and cell-mediated immune responses. The information derived from these studies should contribute significantly to our understanding of normal physiological regulatory mechanisms in immune responses, since both MIF and interferon are physiological mediators which may normally influence development and expression of immune responses.

The development of experimental systems to selectively suppress specific humoral immune responses has direct relevance to control of neoplastic processes, some of which are thought to have cigarette smoking as an etiological component. Antibody responses to tumor antigens often interfere with successful development and expression of cell-mediated immune responses to tumor cells (86, 87). Since a functional cell-mediated immune response is crucial for destruction of the tumor cells, experimental manipulations which eliminate antibody responses to the tumor without compromising cell-mediated immune responses will be especially valuable. It is also highly desirable to suppress antibody responses to only specific antigens, since non-specific suppression of all antibody responses lays the host open to a myriad of other problems. The second portion of this application is directed to this objective.

V. Time Required for This Project

The portion of the project dealing with the characterization and definition of mechanisms of action of Con A-activated suppressor T cells and SIRS will require approximately two years to complete. The portion of the project dealing with generation and characterization of antigen-specific suppressor T cells and their products will require two to three years to complete depending on the rates of success in the initial experiments.

VI. Human Subjects

No human subjects or materials from humans will be used in the proposed studies.

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10. Space and facilities available (when elsewhere than item 2 indicates, state location):

The laboratory facilities are in the Department of Pathology at Harvard Medical School. The research space available for this project is 960 sq. ft. subdivided as follows: semi-isolated tissue culture area, 150 sq. ft.; adjacent walk-in 37°C warm room, 150 sq. ft.; general laboratory area, 570 sq. ft.; and office, 90 sq. ft. Major items of permanent equipment include: refrigerated centrifuges with accessories; microscopes with phase optics; inverted tissue culture phase microscope with photographic accessories; Coulter Counter with size-distribution plotter; Cytograf 6300A for viable cell counting; water jacketed CO_2 incubator; laminar flow tissue culture hoods; pH meter; semi-microbalance; serological water baths; tissue culture incubation chambers; rocker platforms; and refrigerator-freezer.

The Department of Pathology provides the following in support of this project: animal housing facilities; cold rooms; dark rooms; G.E. Maximar 250-III X-irradiation facility; glassware washing and preparation facilities; and tissue processing and histology facilities. The Department also provides the following items of major equipment: spectrophotometers; high speed and ultracentrifuges with rotors; Revco -70°C freezers; gamma and beta scintillation spectrometers; apparatus for programmed freezing of cell suspensions; flourescence, transmission, and scanning electron microscopes and photomicrography equipment; electronic calculating machines and photocopying facilities.

Drs. John David and Heinz Remold of the Robert B. Brigham Hospital will continue to be consultants and active collaborators in aspects of this project involving the characterization of non-specific and antigen-specific suppressor T cell products. Dr. Alice Huang of the Department of Microbiology and Molecular Genetics will be a consultant in aspects of this project involving interferon assays.

11. Additional facilities required: None

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12. Biographical sketches of investigator(s) and other professional personnel (append):

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

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R: REDACTED MATERIAL

12. Biographical Information

Carl William Pierce

Born: REDACTED

Education:

REDACTED A.B., cum laude, Colgate University, Hamilton, New York
M.D., with honors, University of Chicago, Chicago, Illinois
Ph.D., University of Chicago (Pathology), Chicago, Illinois

Research and Professional Experience:

1962 - 1963	U.S.P.H.S. Predoctoral Trainee in Pathology, University
1964 - 1965	of Chicago, Chicago, Illinois
1966 - 1967	Intern and Assistant in Pathology, University of Colorado, Denver, Colorado
1967 - 1970	Research Associate, Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland
1970 - 1973	Assistant Professor of Pathology, Harvard Medical School, Boston, Massachusetts
1973 -	Associate Professor of Pathology, Harvard Medical School, Boston, Massachusetts

Memberships in Professional Societies:

1963
1964
1967
1967
1970
1970

REDACTED REDACTED

Honors and Awards:

1972 - 1977	Recipient of Research Career Development Award from National Institute of Allergy and Infectious Diseases, N.I.H., U.S.P.H.S.

Editorial Boards and Other Appointments:

1972 - 1974	Program Committee, American Association of Immunologists
1974 -	Associate Editor, The Journal of Immunology

Research Interests:

Immunobiology

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13. Pertinent Recent Publications

Carl William Pierce

1. Pierce, C.W., T. Tadakuma, A.L. Kühner, and J.R. David, Characterization of a soluble immune response suppressor (SIRS) produced by concanavalin A-activated spleen cells. *In:* The Role of Mitogens in Immunobiology. J.J. Oppenheim, D.L. Rosenstreich, and M. Landy (Eds.), Academic Press, N.Y., in press.

2. Pierce, C.W., D.L. Peavy, and T. Tadakuma, Suppressor T cells as regulators of lymphocyte functions. Ann. N.Y. Acad. Sci., in press.

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R: REDACTED MATERIAL

12. Biographical Information

Takushi Tadakuma

Born: REDACTED

Nationality:

Education:

Premedical Course, Keio University, Tokyo, Japan
M.D., Keio University School of Medicine, Tokyo, Japan
D.M.S., Keio University Graduate School of Medicine (Microbiology and Molecular Biology), Tokyo, Japan

Research and Professional Experience:

1965 - 1966	Intern, Keio University Hospital, Tokyo, Japan
1966 - 1970	Fellow, Keio University Graduate School of Medicine, Tokyo, Japan
1970 - 1973	Instructor, Department of Microbiology, Keio University, Tokyo, Japan
1973 - Present	Assistant Professor, Department of Microbiology, Keio University, Tokyo, Japan (on leave of absence)
1973 - 1975	Research Fellow, Department of Pathology, Harvard Medical School, Boston, Massachusetts
1975 -	Instructor, Department of Pathology, Harvard Medical School, Boston, Massachusetts

Memberships in Professional Societies:

1967
1967
1971

REDACTED

REDACTED

Research Interests:

Immunobiology

13. Pertinent Recent Publications

1. Pierce, C.W., T. Tadakuma, A.L. Kühner, and J.R. David, Characterization of a soluble immune response suppressor (SIRS) produced by concanavalin A-activated spleen cells. *In:* The Role of Mitogens in Immunobiology. J.J. Oppenheim, D.L. Rosenstreich, and M. Landy (Eds.), Academic Press, N.Y., in press.

2. Pierce, C.W., D.L. Peavy, and T. Tadakuma, Suppressor T cells as regulators of lymphocyte functions. Ann. N.Y. Acad. Sci., in press.

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Takushi Tadakuma

12. Biographical Information

3. Tadakuma, T., T. Mitsuma, *et al.*, *In vitro* reconstitution of anti-sheep erythrocyte antibody response of T cell-depleted spleen cells by allogeneic T cells or factors derived from them. Japan J. Microbiol., in press.

4. Tadakuma, T., Cell cooperation in anti-sheep red blood cell antibody responses in mouse spleen cell cultures: Use of anti-lymphocyte globulin for selective suppression of the antigen reactive cells. Japan J. Microbiol., *16*:287, 1972.

5. Tadakuma, T., K. Saito, *et al.*, Initiation of the primary immune response to sheep red blood cells in the dissociated mouse spleen cell culture. II. Histochemical study on the cell clusters developed during the *in vitro* immune response. Japan J. Microbiol., *15*:493, 1971.

13. Pertinent Recent Publications

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R: REDACTED MATERIAL

12. Biographical Information

Duane Lee Peavy

Born: REDACTED

Education:

REDACTED B.S., with distinction, Ohio State University, Columbus, Ohio
Ph.D., University of Florida (Immunology and Medical Microbiology), Gainesville, Florida

Research and Professional Experience:

1968 - 1972 U.S.P.H.S. Predoctoral Trainee in Immunology and Medical Microbiology, University of Florida, Gainesville, Florida

1973 - Research Fellow, Department of Pathology, Harvard Medical School, Boston, Massachusetts

Memberships in Professional Societies:

1972
1973 REDACTED REDACTED
1973
1975

Honors and Awards:

1968 Graduated with distinction in Microbiology, Ohio State University
1972 Sigma Xi Graduate Student of the Year, University of Florida
1974 - 1976 Recipient of Postdoctoral Fellowship from the National Cancer Institute, N.I.H., U.S.P.H.S.

Research Interests:

Immunobiology

13. Pertinent Recent Publications

1. Peavy, D.L. and C.W. Pierce. Cell-mediated immune responses *in vitro*. III. Elimination of specific cytotoxic lymphocyte responses by ^{3}H-thymidine suicide. Submitted to J. Immunol, 1975.

2. Peavy, D.L. and C.W. Pierce. Cell-mediated immune responses *in vitro*. II. Simultaneous generation of cytotoxic lymphocytes to two sets of alloantigens of limited cross-reactivity. Submitted to J. Immunol, 1975.

3. Pierce, C.W., D.L. Peavy, and T. Tadakuma. Suppressor T cells as regulators of lymphocyte functions. Ann. N.Y. Acad. Sci., in press.

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Duane Lee Peavy

4. Peavy, D.L. and C.W. Pierce, Cell-mediated immune responses *in vitro*. I. Suppression of the generation of cytotoxic lymphocytes by concanavalin A and concanavalin A-activated spleen cells. J. Exp. Med.,*140*:356, 1974.

5. Peavy, D.L., W.H. Adler, J.W. Shands, and R.T. Smith, Mitogenic effect of endotoxin on mouse lymphoid cells: Thymus independence of LPS. Cell. Immunol., *11*:86, 1974.

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14. First year budget:

A. Salaries (give names or state "to be recruited") Professional (give % time of investigator(s) even if no salary requested)	% time	Amount Salary	Fringe Benefits	Total
Carl W. Pierce, Principal Investigator	30			
Takushi Tadakuma, Instructor	100			
Duane L. Peavy, Instructor	100	REDACTED		
Technical				
Research Assistant I, to be recruited	100			
Deborah Siner, Editorial Assistant and Secretary	25	REDACTED		
		REDACTED		
Sub-Total for A				

B. Consumable supplies (by major categories)	
Animals and animal care supplies	3,500
Tissue culture supplies	3,500
Reagents, sera, radioisotopes	2,500
Glassware and general supplies	500
Sub-Total for B	$10,000

C. Other expenses (itemize)	
Domestic travel	500
Portion of service contracts on major equipment shared with other members of Department	300
Publication costs (art work, page costs, reprints)	200
Sub-Total for C	$ 1,000
Running Total of A + B + C	$39,425

D. Permanent equipment (itemize)

None

Sub-Total for D	$ 0
E	$ 5,914
Total request	$45,339

E. Indirect costs (15% of A+B+C)

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2		10,750	1,075	0	7,575	$53,078
Year 3	REDACTED	1,556	1,156	0	8,144	$62,435

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Budget Explanations and Justifications

A. Rationale for this application

This research project has been developed both from the results of other investigators and from experiments in my laboratory currently supported by grants from the National Institutes of Health (see item 16). Insufficient funds are available in these grants to pursue the proposed project in the desired fashion without compromising the progress of other important projects. Further, since some of the objectives of the proposed project are outside the major objectives of these grants, it is inappropriate to continue to support the proposed project entirely with funds from these grants. Our experience with the experimental approaches and methodology to be used have demonstrated that this project is feasible and will provide significant new and relevant information about the mechanisms by which suppressor T cells and their products regulate humoral and cell-mediated immune responses.

B. Personnel

This project, as conceived, will require the full-time efforts of the two Instructors, who are currently actively involved in various aspects of the project, and a Research Assistant. The Principal Investigator will devote at least 30% of his time to the supervision of the progress of the project and actual experimentation. Dr. Tadakuma, Instructor, is on leave of absence from Keio University Medical School, Tokyo, Japan, which provides approximately half his salary. Funds are requested for the remainder of his salary and fringe benefits (16%, on that portion of salary requested only) to bring his total remuneration to a level commensurate with other Instructors with comparable training and experience. Dr. Duane L. Peavy will be supported by an N.I.H. Postdoctoral Fellowship until June 30, 1976, at which time he will be appointed Instructor. Funds for salary and fringe benefits (16%) from July 1 to December 31, 1976 are requested. The increased salary budget in years 2 and 3 reflects, in part, Dr. Peavy's salary on a 12-month basis. The continuation of these two Instructors, who are presently involved with aspects of this project, will guarantee uninterrupted progress of experimentation. Since this grant, if funded, will support approximately 25% of the research activities of the laboratory, funds are requested for 25% of the salary and fringe benefits (14.5%) of Ms. D. Siner, Editorial Assistant and Secretary, who is presently supported entirely by the listed N.I.H. Grants. Fringe benefits for the Research Assistant are 14.5%. An annual salary increase of 7.5% for personnel is requested.

C. Consumable Supplies

Funds requested in each category are estimated from current operating expenses for those portions of the project already in progress and are a realistic estimate of the funds necessary to carry out the proposed project in the desired fashion. An annual increase of 7.5% for supplies is requested.

D. Other Expenses

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Travel funds to enable Drs. Tadakuma and Peavy to attend one scientific

Source: https://www.industrydocuments.ucsf.edu/docs/rpdm0000

meeting per year are requested. Funds for a portion of service contracts on major equipment shared with other members of the Department are requested. Based on our experience with the proposed projects, publications in the first year are almost assured, and therefore funds for publication costs (page charges, art work, and 200 reprints) are also requested. An annual increase of 7.5% in this category is requested.

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Source: https://www.industrydocuments.ucsf.edu/docs/rpdm0000

16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
1. Cell Interactions in Immune Responses *in Vitro*	National Institutes of Health Research Grant AI-09897	$204,050	9/1/73 - 8/31/78
		$ 40,000	9/1/75 - 8/31/76
2. Studies on Experimental Cancer Immunology, Project V: Studies on Tumor Specific Immunity *in Vitro*.	National Institutes of Health Program Project Grant CA-14723	$134,744	6/30/73 - 5/31/78
		$ 26,177	6/1/75 - 5/31/76

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Biology of Suppressor T Cells	American Cancer Society (pending)	$ 86,373	1/1/76 - 12/31/77
		$ 42,509	1/1/76 - 12/31/76

(The application to the A.C.S. is similar, but not identical, to the present application. If both applications should be funded, only one award would be accepted.)

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Carl W. Pierce

Signature Carl W. Pierce Date 6/25/75

Telephone 617-734-3300, Extension 337
Area Code Number Extension

Checks payable to
Harvard Medical School, c/o Business Office

Mailing address for checks
25 Shattuck Street
Boston, Massachusetts 02115

Responsible officer of institution

Typed Name Henry C. Meadow

Title Executive Secretary, Committee on Research and Development

Signature _____ Date _____

Telephone 617 - 734-3300, Extension 441
Area Code Number Extension

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Source: https://www.industrydocuments.ucsf.edu/docs/rpdm0000